BACTERIOPHAGE THERAPY: AN ALTERNATIVE TO ANTIBIOTIC THERAPY IN AQUACULTURE?

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Bacteriophage Therapy: An Alternative to Antibiotic Therapy in Aquaculture?

by

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ABSTRACT

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The Newfoundland salmonid-aquaculture industry has faced many challenges since its inception in the mid-1980s. Of particular concern to the Newfoundland Salmonid Growers Association (NSGA) over the past decade is *Aeromonas salmonidica* subspecies *nova*, the bacterial agent responsible for atypical furunculosis in Bay d'Espoir. Although improvements to industry husbandry practices throughout the late 1990s were instrumental in bringing mortality attributable to this pathogen under control, the salmonid farmers have been anxious to enhance the veterinary tools available to them in the event that the pathogen should become troublesome at some time in the future. To this end, the NSGA was successful in launching a four-year study of the molecular biochemistry, infectivity and pathogenicity of the strain of the atypical-furunculosis bacterium found in the Bay d'Espoir fjord.

This report documents one aspect of the atypical furunculosis research program, namely a literature review of bacteriophage treatment, either by itself or in combination with other approaches to disease intervention. Based on published reports of investigations of the potential for bacteriophages to control bacterial diseases of farmed fish, this review concludes:

- that within the limits of our current understanding, the properties of the phages of bacterial pathogens of fish are identical to those of phages of bacterial pathogens of terrestrial animals, including humans;
- that the comparison of antibiotics and bacteriophages as therapeutants of bacterial diseases of terrestrial animals also applies to the use of these agents against bacterial pathogens of aquacultured finfish;
- that the use of bacteriophages to treat bacterial diseases of aquacultured species has the added advantage of reducing the environmental load of the pathogen as phages are as effective in the environment as they are in the fish; and,
- that the combined characteristics of the bacteriophages as therapeutic agents for bacterial diseases of aquacultured species indicate that there should be a serious effort to assess their potential using various models of bacterial diseases of salmonids.

RÉSUMÉ

Morrison, S., and Rainnie, D.J. 2004. Bacteriophage therapy: an alternative to antibiotic therapy in aquaculture? Can. Tech. Rep. Fish. Aquat. Sci. 2532: v + 23 p.

L'industrie de l'aquaculture des salmonidés à Terre-Neuve a fait face à de nombreux défis depuis son lancement, au milieu des années 1980. Au cours de la dernière décennie la Newfoundland Salmonid Growers Association (NSGA) s'est tout particulièrement inquiétée de la sous-espèce *nova* de *Aeromonas salmonidica*, l'agent bactériologique responsable de la furonculose atypique dans la baie d'Espoir. Quoique les améliorations apportées aux techniques d'élevage utilisées par l'industrie à la fin des années 1990 aient contribué à enrayer la mortalité attribuable à cet agent pathogène, les éleveurs de salmonidés attendent impatiemment de pouvoir disposer d'outils vétérinaires pour le cas où cet organisme pathogène leur causerait à nouveau des problèmes à l'avenir. C'est pourquoi la NSGA a entrepris une étude de quatre ans portant sur la biochimie moléculaire, sur l'infectiosité et sur la pathogénicité de la souche de bactéries responsable de la furonculose atypique qu'on trouve dans le fjord de la baie d'Espoir.

Le présent rapport documente un aspect du programme de recherche sur la furonculose atypique, en l'occurrence une analyse bibliographique du traitement par bactériophages, utilisé soit seul, soit en combinaison avec d'autres moyens de lutte contre la maladie. En se fondant sur les rapports qui ont été publiés au sujet du potentiel qu'offrent les bactériophages dans la lutte contre les maladies bactériennes du poisson d'élevage, on en arrive aux conclusions suivantes :

- Dans les limites de nos connaissances actuelles, les propriétés des phages des bactéries pathogènes du poisson sont identiques à celles des phages des bactéries pathogènes des animaux terrestres et des humains;
- La comparaison entre les antibiotiques et les bactériophages comme agents curatifs des maladies bactériennes chez les animaux terrestres s'applique aussi à leur utilisation contre les bactéries pathogènes des poissons d'élevage;
- L'utilisation de bactériophages pour traiter les maladies bactériennes des poissons d'élevage offre en plus l'avantage de réduire la charge environnementale en agent pathogènes, les phages étant aussi efficaces dans l'environnement qu'ils le sont dans le poisson;
- Compte tenu des caractéristiques combinées des bactériophages comme agents thérapeutiques dans les maladies bactériennes des poissons d'élevage, il conviendrait de consacrer sérieux efforts à l'évaluation de leur potentiel, en utilisant divers modèles de maladies bactériennes des salmonidés.

INTRODUCTION

The Bay d'Espoir salmonid-aquaculture industry has faced many challenges since its inception in the mid-1980s. While many of these challenges have been overcome through refinements to husbandry practices and a better understanding of salmonid physiological response to the estuarine-fjord environment, one challenge that has been particularly persistent is the naturally-occurring fish pathogens of the area. Of particular concern to the Newfoundland Salmonid Growers Association (NSGA) over the past decade is *Aeromonas salmonidica* subspecies *nova*, the bacterial agent responsible for the atypical furunculosis of salmonids as experienced in Bay d'Espoir. The salmonid farmers have been anxious to enhance the veterinary tools available to them in the event that this pathogen should become troublesome at some time in the future. To this end, the NSGA approached the Government of Newfoundland and Labrador, Department of Fisheries and Aquaculture, and the Government of Canada, Department of Fisheries and Oceans for assistance in implementing a long-term, proactive approach to their fish-health management needs. As a result of this collaboration, and with the involvement of the finfish health research resources of the Atlantic Veterinary College, the NSGA was successful in launching a four-year study of the biochemistry, infectivity and pathogenicity of the strain of the atypical furunculosis bacterium found in the Bay d'Espoir fjord.

Although much research has been done on furunculosis of salmonids, the NSGA was anxious to quantify the efficacy of existing furunculosis treatments and determine if development of new fish-health intervention tools is warranted. Accordingly, the research program undertaken in this collaboration of fish farmers, veterinarians and scientists was structured to characterise the nature of the pathogen itself, investigate possible alternative treatments for controlling the pathogen, and the potential for development of new vaccines should the efficacy of existing vaccines prove limited. This report documents one aspect of the research program, namely investigation of bacteriophage treatment, either by itself or in combination with other approaches as a means of disease intervention.

HISTORY OF BACTERIOPHAGE RESEARCH

Bacteriophages are viruses that invade bacterial cells (Sulakvelidze *et al.* 2001). Their discovery and application to the treatment of infectious diseases of man and terrestrial animals pre-dates antibiotics. Bacteriophages were discovered independently in 1915 by Frederick Twort and in 1917 by Felix d'Herelle (Lederberg 1996; Barrow and Soothill 1997; Alisky *et al.* 1998; Carlteon 1999; Barrow 2001; Chanishvili *et al.* 2001; Ho 2001; Sharp 2001; Sulakvelidze *et al.* 2001; Summers 2001). Twort did not pursue his findings, but d'Herelle and his colleagues used phages in various settings on a variety of species, from chickens to humans. The results were positive from a clinical assessment, successfully treating diseases from avian typhosis to bubonic plague (Ho 2001; Sharp 2001; Sulakvelidze *et al.* 2001; Summers 2001). However, from a

scientific perspective, these trials often were poorly designed and poorly described. The early trials on phage therapy lacked controls, failed to provide information on size and spacing of doses or routes of administration, and provided no information on the production of the phage preparation (Shultz 1932 as cited in Ho 2001; Barrow and Soothill 1997; Carleton 1999; Summers 2001). This information is summarized in Table 1.

Application of bacteriophages to the treatment of infectious diseases has been centered in Eastern Europe and the countries of the former U.S.S.R. The effectiveness of phages was controversial, particularly in the Western world. Once antibiotics were discovered, interest in phage therapy waned (Alisky *et al.* 1998). Two centres of phage therapy research for human diseases persisted in Soviet-bloc countries through the twentieth century, the Eliava Institute founded in 1923 in Tbilisi, Georgia, and the Hirszfeld Institute in Poland, founded in 1952. Much of the research from these Institutes was clinical in nature and lacked scientific rigor (Sulakvelidze *et al.* 2001).

In the 1980s, increasing problems with antibiotic-resistant bacteria prompted researchers to reconsider bacteriophage therapy as an alternative to antibiotics (Merril *et al.* 1996; Chopra *et al.* 1997; Carleton 1999; Ho 2001). Since then, studies have been carried out in Poland, the former Soviet Union, Great Britain and the USA. These studies were conducted on a host of terrestrial species, ranging from chickens to humans. Phage therapy was found to be effective against many different genera of bacteria. These studies are summarized in Appendix A. Some of this research is reviewed below. For more detailed reviews, the reader is directed to Barrow and Soothill (1997), Alisky *et al.* (1998), Carleton (1999), Chanishvili *et al.* (2001), Sulakvelidze *et al.* (2001) and Summers (2001).

Slopek *et al.* (1987) reported on the application of bacteriophage therapy in Poland in 550 cases of bacterial infection in humans, ranging in age from 1 week to 86 years, between 1981 and 1986. These infections were caused by pyogenic *Staphylococcus* spp. and Gram-negative bacteria (*Klebsiella* spp, *Escherichia coli* spp, *Proteus* spp and *Pseudomonas* spp). Bacteriophage preparations were administered orally three times per day before meals in the form of 10 mL of phage suspension, after neutralization of gastric juices with baking soda or bicarbonated mineral water. In some cases, phages were also applied locally as moist dressings to wounds, or to pleural and peritoneal cavities, to the urinary bladder and as eye, ear and nose drops. Positive results were reported in 508 cases (94%). Thirty-eight cases (6.9%) exhibited transient improvement, and four cases (0.7%) showed no improvement. In 518 of these cases, bacteria were indicated to be antibiotic-resistant but the antibiotics were not identified. Control groups without phage therapy were not included in the study. Rarely were any side effects reported.

Table 1. Some of the problems with early therapeutic phage research and the ways they have been addressed in more recent studies or could be addressed in the future. (Modified from Sulakvelidze *et al.* 2001).

Problem	Comments	Solution and/or required approach
Narrow host range of phages	Because of the high specificity of phages, many negative results may have been obtained because of the failure to select phages lytic for the targeted bacterial species.	Determine the phage susceptibility of the etiologic agent before using phages therapeutically; use polyvalent phage cocktails which lyse the majority of strains of the etiologic agent.
Insufficient purity of phage preparations	Early therapeutic phages were in crude lysates of host bacteria. Their preparations contained numerous contaminants (including endotoxins) that may have counteracted the effectiveness of phages.	Ion-exchange chromatography, high-speed centrifugation, and other modern purification techniques should be used to obtain phage preparations of high purity.
Poor stability and/or viability of phage preparations	Some commercial phage preparations were supplemented with mercurials or oxidizing agents or were heat-treated to ensure bacterial sterility. Many of these treatments also may have inactivated the phages, resulting in ineffective phage preparations.	Advanced purification techniques can be used to purify phages and to ensure that they are bacterium free. The viability and titre of phages should he determined before using them therapeutically.
Lack of understanding of the heterogeneity and mode of action of phages (i.e. lytic vs lysogenic phages)	Failure to differentiate between lytic and lysogenic phages may have resulted in some investigators using lysogenic phages, which are much less effective than lytic phages.	Carefully select for lytic phages. This also is critical for avoiding the possible horizontal transfer of genes for bacterial toxins, antibiotic resistance, etc., by lysogenic phages.
Exaggerated claims of effectiveness of commercial phage preparations	One example of this would be the preparation called *Enterophagos*, which was marketed as being effective against herpes infections, urticaria and eczema, conditions against which phages could not possibly be effective.	Phage preparations should be accompanied by specific, scientifically supported information about their efficacy against specific bacterial pathogens and indications of their possible side-effects.
Failure to establish scientific proof of efficacy of phage treatment	Most clinical studies using therapeutic phages were conducted without placebo controls; when placebo controls were used, data were evaluated in a subjective manner questioned by many peers.	Carefully controlled, double-blinded, placebo-controlled studies with highly purified, lytic phages should be conducted and results must be evaluated based on both clinical observations and scrupulous laboratory analysis.

One of the most extensive clinical evaluations of the efficacy of bacteriophage therapy in humans was a study conducted in Tbilisi, Georgia from 1963 to 1964, using bacteriophages of *Shigella* spp. as a prophylaxis in the control of dysentery in children (Babalova *et al.* 1968, as cited in Sulakvelidze *et al.* 2001). This 109-day investigation involved 30,769 children between the ages of 6 months and 7 years. The children on one side of the streets (17,044 children) were given dried *Shigella* bacteriophages orally once every 7 days, whereas the children on the other sides of the streets (13,725 children) did not receive phages. The children were monitored weekly at the time of receiving the phages. Fecal samples were collected from all children with gastrointestinal disorders to be cultured for the presence of *Shigella* spp. and other diarrhea- causing bacteria. Clinically, the incidence of dysentery was 3.8-fold greater in the untreated children. Based on bacterial-culture confirmed cases, the incidence of dysentery was 2.6-fold greater in the untreated children. An interesting outcome of the study was that there was an overall 2.3-fold reduction in diarrheal diseases of unknown origin among children treated with the phages relative to the untreated group. The reason for this is unknown but may be due in part to activity of the phage preparation towards bacterial gastrointestinal pathogens other than *Shigella* spp.

In most of the studies from Poland and the former Soviet Union, the effectiveness of phage therapy was not questioned and consequently the use of untreated, or placebo controls was rare. It is with the more recent research of Smith and Huggins (1982, 1983), Smith et al (1987a, and b), and Soothill (1992) from Britain, and Geier et al. (1973) and Merril et al. (1996) from the United States of America, that bacteriophage efficacy has been investigated in a more rigorous scientific manner.

Smith and Huggins (1982) used a generalized *E. coli* infection of mice following intra muscular (i.m.) and intra cerebral injection of MW-strain *E. coli* to investigate the comparative efficacy of the bacteriophage anti-K1 and various antibiotics administered i.m. They found that the survival of mice infected i.m. with *E. coli* and given one dose of phage 8 h later was significantly greater than the survival of *E. coli* infected mice given either one dose or eight doses of either tetracycline, ampicillin, chloramphenicol, or equal parts trimethoprim and sulphafurazole, or one dose of streptomycin. The isolate of the MW strain of *E. coli* used in this study was sensitive, as determined by *in vitro* minimum inhibitory concentrations, to all of the antibiotics investigated. Survival rate of groups of mice infected intra cerebrally with *E. coli* and then given phages i.m. 16 days later was significantly greater than that of groups of mice given 12 doses of either tetracycline, ampicillin, chloramphenicol or a mixture of trimethoprim and sulphafurazole. The survival of those treated with streptomycin did not differ significantly from those that were phage-treated.

Smith *et al.* (1987a and b) found that experimentally-induced *E. coli* diarrhea in calves could be cured by a single injection of 10^5 plaque-forming units (pfu) of bacteriophage. They also found that *E. coli* infection could be prevented by doses as low as 10^2 pfu if sprayed as an aqueous suspension on the litter in calf rooms. In some of the trials where calves were infected with a single strain of *E. coli*, a phage-resistant

strain of *E. coli* emerged. However, this bacterium lacked the specific K antigens on the outer surface that are required for phage attachment and which also are known to be involved in the pathogenicity of the *E. coli*. Consequently the phage-resistant mutant was considerably less pathogenic than the wild K+ type.

In another study calves were given a mixture of six *E. coli* strains that were shown to be controlled by a mixture of the six phages specific to the bacteria. However, this control was not as complete as that seen in single-strain infections. In this trial, K+, phage-resistant bacteria emerged, which were as virulent as the inoculated strains. The authors stated that these phage-resistant bacteria could be controlled by mutant phages derived from those that were active on the original strains. They demonstrated that success in treating *E. coli* infections depended on the dosage and timing of administration. Doses of 10^5 viable bacteriophages given to calves six hours before they were infected with 10^9 viable *E. coli* protected the calves from diarrhea whereas a lower dose of phage, 10^2 pfu, did not provide protection when given six hours before *E. coli*. The lower dose did protect when given 10 minutes before, or at 6 or 12 hours after *E. coli* inoculation (Smith *et al.* 1987a). The authors believed this study showed that phage could be used in the field to control outbreaks of *E. coli* enteritis. This research group also found positive results in lambs and piglets (Smith and Huggins 1983).

Soothill (1992) investigated phage therapy against three species of bacteria for which antibiotic resistance frequently is a problem: *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Acinetobacter baumanii*, using mice as the experimental model. Bacteria and phage were injected into the abdomen of outbred CBA mice (Jackson Laboratories of Bar Harbour, Maine; http://www.informatics.jax.org/external/festing/mouse/docs/CBA.shtml accessed 17/11/2003). As few as 10^2 acinetobacter-phages, BS46, were sufficient to protect mice against 5 x LD_{50} (1 x 10^8 cfu) of *A. baumanii*. A pseudomonas-phage, BS24, protected mice against 5 x LD_{50} of *Pseudomonas aeruginosa*. However, a staphylococcal-phage, of the staphylococcal bacterial strains used in the challenge, φ-131, that was poorly lytic *in vitro*, did not protect mice from *S. aureus* challenge. The numbers of phages detected in the tissues of surviving animals were far in excess of those given, indicating replication *in vivo* during the bacterial killing process.

BACTERIOPHAGE VERSUS ANTIBIOTICS

Table 2 summarizes a comparison of some of the characteristics of bacteriophages and antibiotics as therapeutic agents. The characteristics presented relate to achieving and maintaining effective therapeutic concentrations of the agents, specificity of action, and the development and resolution of bacterial resistance to the agents.

Differences in two of the characteristics are worth emphasizing. Phages need to be given only as one dose. Their number will increase over time at the site of the

infection, whereas antibiotic concentration is not targeted to the site of infection and will decrease over time (Smith and Huggins 1983). Antibiotic therapeutic success depends on intervals of administration, the concentration of antibiotic employed, and antibiotic concentration decay rates (Levin and Bull 1996) and when possible, selection of an antibiotic that has a preferential concentration in the organ or tissues affected by the infection. If the concentration of an antibiotic declines below bactericidal levels to concentrations permissive for bacterial growth, the bacterial population could recover and reach the threshold concentrations required to cause morbidity or death before the bacteria are controlled by the immune response. The occurrence of antibiotic-resistant bacteria is increasing and frequently there is an increased pathogenicity associated with antibiotic resistance. Phage-resistant bacteria that have emerged in phage-treated animals often were less virulent than the parent strain of bacteria.

CHALLENGES TO THE IMPLEMENTATION OF BACTERIOPHAGE THERAPY

Challenges to implementation of bacteriophage therapy in humans and other terrestrial homeotherms have been discussed in some detail by Carlton (1999) and have been identified also by Alisky *et al.* (1998). The challenges, proposed solutions and considerations in applying phage therapy to bacterial pathogens of aquatic species are discussed briefly below.

Host range

Bacteriophages have a relatively narrow host range limited to a particular species of bacteria and frequently only to a greater or lesser proportion of the strains of this bacterium. It is important that the bacterial strain one intends to treat with a bacteriophage be susceptible to the bacteriophage preparation to be used, and that the phage should be strongly lytic in the bacterial isolate. These requirements can be achieved by:

- screening the bacterial isolate against a panel of bacteriophages for the bacterial species of concern, much in the way that antibiotic culture and sensitivity currently are conducted;
- selecting bacteriophages with broad across-strain lytic activity; and
- developing a 'multivalent' phage preparation that lyses all or most of the strains within a species of bacterial pathogen.

An additional consideration for bacteriophages of bacterial pathogens of poikilotherms is that the phages must demonstrate a high rate of replication and lysis of the bacterium in the temperature range within which the bacterial species causes the disease problem.

Table 2. A comparison of antibiotics and bacteriophages as therapeutants for bacterial infections. (Modified from Carlton (1999) and Sulakvelidze *et al.* (2001)).

The Issue	Antibiotics	Bacteriophages
The fate of the administered therapeutant	Metabolic destruction of the therapeutant and elimination from the patient.	Exponential growth in numbers so that the therapeutant propagates at the site(s) of the infection where it is required.
The concentration of the therapeutant required to kill a given bacterium, and the change in the concentration of the therapeutant between treatments	Numerous molecules of the antibiotic are needed to kill a bacterium. The uptake of the antibiotic by the bacterium is a passive, diffusion-driven process. During initiation of therapy and between administrations of successive doses, the sub-lethal concentration that bacteria experience affords an opportunity for replication of that portion of the bacterial population with a lower sensitivity to the therapeutant (selection for resistance).	One phage particle is sufficient to kill a bacterium sensitive to the phage. It is suggested that the rate of development of resistance to bacteriophages is one-tenth (Carleton 1999) the rate of development of resistance to antibiotics.
Response to bacterial resistance	There is no inherent ability of chemicals to respond to development of resistance by the bacterial pathogen. The response has to be one by the clinician in modifying the therapy, either by increasing the dose rate of the antibiotic, if that is safe, or by changing the antibiotic. Development of a new class of antibiotic (to address the problem of multiple antibiotic resistant bacteria) requires several years and is an expensive process.	Bacteriophages are biological 'organisms' or entities capable of undergoing mutations, some of which can overcome the bacterial mutation to resistance to the original phage. Selection of new phages (e.g. against phage-resistant bacteria is a relatively rapid process that frequently can be accomplished within days or weeks.
Spread of bacterial resistance	Antibiotics used in the treatment of bacterial infections tend to be broad spectrum, thereby selecting for resistance in several species of bacteria (in addition to the one targeted). Since mechanisms of antibiotic resistance usually occur at the level of enzymatic processes or of the cell membrane, properties which are shared by many bacterial species, it is possible for the resistance to an antibiotic to be spread rapidly from one species of bacterium to another through the transfer of genetic information (e.g. plasmid transfer).	Bacteriophages tend not to cross bacterial species boundaries. Thus even though the targeted bacterial species may become resistant to the phage, should nucleic acid be transferred from this species of bacterium to another species, it is unlikely that this will in any way influence the sensitivity of the recipient species of bacterium to its specific phages
Activity against non-target bacterial species	The administration of broad-spectrum activity antibiotics, particularly for prolonged periods or at elevated dose rates, can eliminate normal flora, particularly of the gastrointestinal tract and predispose the patient to infections by opportunistic bacterial and fungal pathogens.	The narrow host range of bacteriophages avoids this problem.

Lysogeny

There are two types of bacteriophage reproduction:

1) *Lytic* - The virus attaches to a host cell and injects its nucleic acid into the cell, directing the host to produce numerous progeny. These then are released by a fatal bursting of the cell, allowing the cycle to begin again;
2) *Lysogenic* - The nucleic acid of the virus becomes part of the host genome and reproduces genetic material (prophage) in the host cell. An induction event, such as a physiological stressor, can trigger this reproduction to switch to lytic (Fuhrman 1999) (Fig. 1).

Lysogenic bacteriophages may incorporate into the genome of the bacterium rather than being lytic. These phages are poor candidates for therapy as they do not provide the rapid growth in phage numbers and associated bacterial lysis that is required to be effective and reliable as a therapy.

In order to be effective against bacterial pathogens, phages selected for treatment must be lytic, disrupting bacterial metabolism and reproduction and causing the bacterium to lyse (Sulakvelidze *et al.* 2001). Only lytic phages should be considered as candidates for bacteriophage therapy. The DNA of bacteriophages which are strong candidates for therapy should be sequenced for homologies to known genes of lysogeny.

Bacterial debris in bacteriophage preparations

The presence of even small quantities of endotoxin or other bacterial debris can be fatal to the patient if administered parenterally. Removal of bacterial debris from bacteriophage preparations is readily achieved by current technologies of density centrifugation and various forms of column chromatography. Bacterial debris in the phage preparation would be of somewhat less concern in the treatment of most bacterial pathogens of aquatic species as the route of administration will be oral in the vast majority of instances.

Attempts to ensure the sterility of therapeutic bacteriophage preparations

During early investigations of the effectiveness of bacteriophage therapy, agents used in an attempt to ensure that the phage preparation did not contain live bacteria (*e.g.* mercurials, oxidizing agents and heat) had significant potential to inactivate the bacteriophages as well.

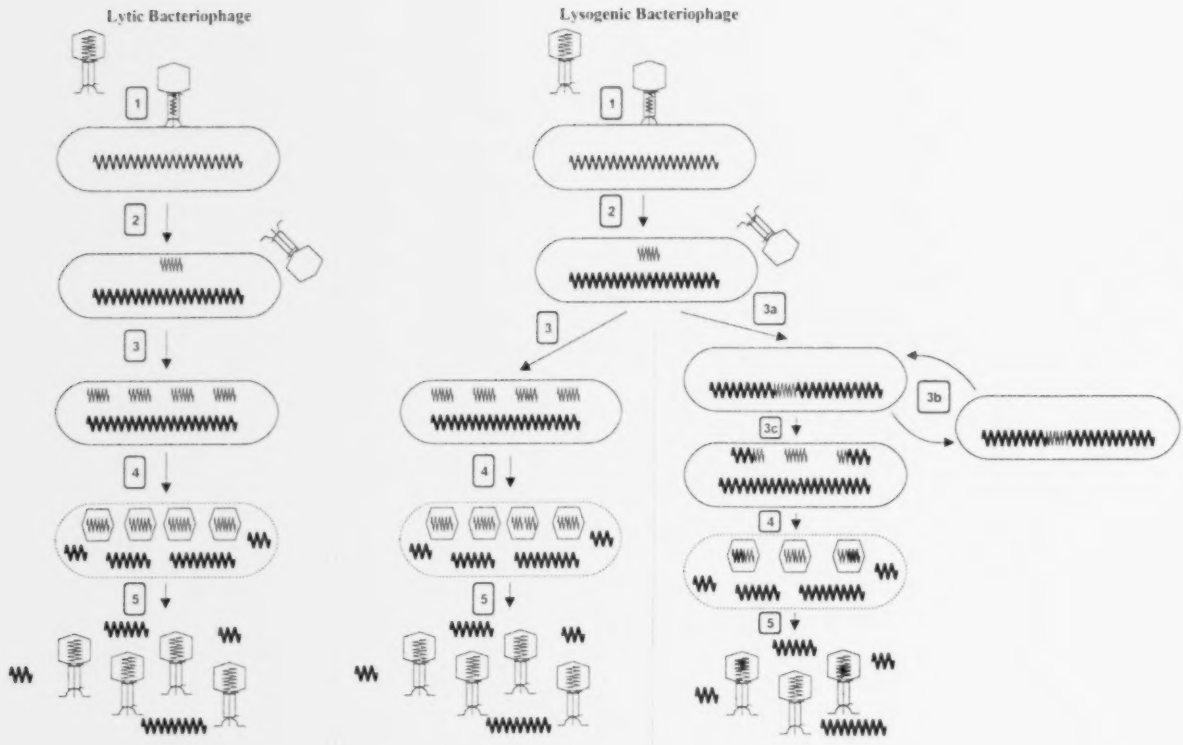

Figure 1. Replication cycles of lytic and lysogenic bacteriophages.

Lytic bacteriophages: (1) attachment to a susceptible bacterial host; (2) injection of bacteriophage nucleic acid into the susceptible bacterial host; (3) shut-down of synthesis of components of the host bacterium, replication of the phage nucleic acid and the production of phage proteins; (4) assembly of bacteriophages; (5) bacterial cell lysis and the release of mature bacteriophages.
Lysogenic bacteriophages: (1) attachment to a susceptible bacterial host; (2) injection of phage nucleic acid into the susceptible bacterial host; Following the injection of the phage nucleic acid, lysogenic phages can initiate a replication cycle similar to that of lytic phages (3), or they can integrate their genome into the host bacterium's chromosome (lysogenization) (3a). Lysogenized bacteria may replicate normally for many generations (3b) or at some point may undergo induction (3c), spontaneous or physical or chemical agent induced, at which time the integrated phage genome is excised from the bacterial chromosome and may pick up fragments of bacterial DNA in the process. The bacteriophages are assembled (4) and the bacterial cell lyses releasing the mature bacteriophages (5).

This problem can be solved by sterile filtration of the bacteriophage preparation. Alternatively, for those phage preparations to be administered *per os*, if the phage is selected for its ability to lyse a broad spectrum of bacterial strains, it may be possible to select a non-pathogenic bacterial strain for the production of the phage. If it proves possible to produce the phages of interest in non-pathogenic strains, complete removal of all the host bacteria would not be a concern for oral administration. In fact if the production in non-pathogenic strains were possible, it may be of value to use intact non-pathogenic bacteria, infected with the phage, as a means of circumventing or reducing

the inactivation of some bacteriophages experienced when they are administered *per os* as an unprotected phage preparation. This approach is supported by the observations of Smith *et al.* (1987b) who reported in phage therapy of calves that the phage was more susceptible than its target bacteria to acid lysis.

Rapid clearance of bacteriophages by the reticuloendothelial system (RES)

Investigations by Geier *et al.* (1973) demonstrated that when administered parenterally to gnotobiotic animals, bacteriophages were rapidly cleared from the circulation. The use of gnotobotic animals ensured that the clearance was not due to the presence of pre-existing neutralizing antibodies to the bacteriophages. Subsequent observations revealed that the phages were not engulfed by macrophages. The phages were sequestered passively by the RES of the spleen and consequently were prevented from reaching the site of bacterial infections.

As a solution for this problem, Merril *et al.* (1996) showed that it was possible, through serial passage of the bacteriophages of interest in the target host, to select phages that had a much reduced sequestration by the host's RES. This research demonstrated also that the phages with the lower sequestration were superior in the treatment of a fulminant bacteremia.

Anti-bacteriophage antibodies

A concern in the application of bacteriophages to the treatment of bacterial infections is the development of neutralizing antibodies to the phage by the host animal. Since the process of development of neutralizing antibodies takes a few weeks in homeotherms, neutralizing antibodies are unlikely to be a problem in the application of bacteriophage therapy to acute bacterial infections. However, neutralizing antibodies could be a problem for prolonged phage therapy of chronic bacterial infections, or in cases of repeated phage therapy in recurrence of the same bacterial infection.

When attempting phage therapy of chronic or recurrent bacterial infections, it may be possible to compensate for the affect of neutralizing antibodies by administering a greater dose of phage. It is recommended when applying bacteriophage therapy under these circumstances that the type and titre of the antibodies that develop be monitored (Carlton 1999).

Historical reputation of bacteriophage therapy

There is considerable skepticism about the potential of phage therapy in the treatment of bacterial diseases. Much of this skepticism is based on poor quality of the science of the early clinical trials to determine efficacy of bacteriophages in the treatment of bacterial diseases of humans and terrestrial animals. The research

conducted failed to document the sources and the methods of production of the bacteriophage preparations, the characteristics of the phage preparations such as volume administered and the concentration of phage particles, and the frequency of dosing. Particularly important to critical scientific assessment was the failure to conduct trials as double-blind, placebo-controlled studies.

However, the successes achieved more recently in well controlled, experimental studies with animal models of human disease, and in clinical trials for the treatment of animal diseases, provides strong support for bacteriophage therapy as an alternative to antibiotic therapy in the treatment of bacterial diseases. It is critical that all future investigations in this area be conducted to rigorous standards of study design, conduct, documentation and bias control.

APPLICATION OF BACTERIOPHAGE THERAPY IN AQUACULTURE

A limited number of licensed antibiotics are available in Aquaculture. At present, antibiotics such as oxytetracycline, potentiated sulfonamides, fluoroquinolones and florfenicol are administered primarily in the feed. However, due to poor feed consumption by diseased fish, environmental factors and the occurrence of antibiotic resistant strains, this chemotherapeutic method is not always successful (Wu and Chao 1982).

To date, there have been only a few attempts to use phages for disease control in aquaculture. One of the earliest investigations of the potential application of phages to aquaculture was reported by Wu and Chao in 1982. They examined the effect of a phage, φET-1, isolated from pond water in Taiwan, on *Edwardsiella tarda*. *In vitro*, φET-1 had a wide spectrum of host *E. tarda* strains, killing 25 of 27 strains (92.6%) of *E. tarda* strains exposed, which demonstrated its potential for control of the disease. When a bacterial suspension of 1.2×10^9 cells/mL was infected with φET-1 at a multiplicity of infection (M.O.I) of 0.08, the surviving bacteria were less than 0.1% of the starting concentration after 8 h. During this time period, the number of plaque-forming units of the bacteriophage increased from 1×10^8 pfu/mL to 1×10^9 pfu/mL. This experiment showed that *E. tarda* could be killed in the water system, allowing for an economical method of disease control.

In a second experiment, Wu and Chao (1982) examined the effect of φET-1 infected, *E. tarda* suspension on loaches (*Misgurnus anguillcaudatus*). *E. tarda* was suspended in tap water at 1×10^8 cfu/mL and inoculated with bacteriophage φET-1 at a M.O.I. of 0.1. At different times post-phage φET-1 inoculation, loaches were immersed in the φET-1 infected, *E. tarda* suspension for one hour and subsequently monitored for mortalities. Exposure of loaches to the φET-1 infected, *E. tarda* suspension two minutes after addition of the phage φET-1 resulted in mortality similar to that obtained with *E. tarda* suspensions that had not been inoculated with φET-1 (i.e. 100% mortality of the loaches within 48 h of exposure). Exposure of loaches to the φET-1 infected, *E. tarda* suspension two hours after the φET-1 inoculation, resulted in a 5% survival of

loaches for more than 4 days. Exposure of loaches to the φET-1 infected, *E. tarda* suspension eight hours after φET-1 inoculation of the *E. tarda*, resulted in 90% survival of the loaches. The longer the time post φET-1 infection of bacteria, the better the protection against infection, due to the multiplication of phage.

A complete growth cycle for phage consists of adsorption to host cell surface, injection of nucleic acid, replication of nucleic acid, assembly of virion, and release of bacteriophage progeny by lysis of the bacterial cell. Total cycle time is usually 1-2 h at 30°C. *E. tarda* suspensions exposed to φET-1 for 2 minutes still contained approximately 90% live bacteria. Consequently pathogenicity showed up as quickly as in the *E. tarda* suspensions that had not been inoculated with φET-1. However, after eight hours of φET-1 infection, the phage would have undergone several cycles of replication with the result that most of the bacteria were lysed. The authors concluded that φET-1 had a broad spectrum of bacterial-strain infectivity, rapid killing of host bacterial cells, continuous replication of phages and effective elimination of *E. tarda* pathogenicity. They concluded this could be a powerful tool for controlling *E. tarda* in pond water.

Bacteriophages were investigated first as a treatment for bacterial diseases of cultured finfish on *Lactococcus garvieae* (formerly known as *Enterococcus seriolicida* or *Streptococcus* sp.) infections of yellowtail (*Seriola quinqueradiata*) (Park *et al.* 1997, 1998; Nakai *et al.* 1999). *Lactococcosis* has caused serious economic damage to yellowtail aquaculture in Japan, particularly because of its occurrence in market-size fish when the water temperature exceeds 20°C.

Park *et al.* (1997) initially recovered a virulent bacteriophage of *L. garvieae* from a culture of the bacterium isolated from diseased yellowtail. Initial investigations demonstrated that this was a double-strand, DNA phage with a broad range of infectivity to *L. garvieae* strains (24/26) but very high host bacterial-species specificity, having no ability to infect 22 other species of aquatic bacteria pathogenic to finfish and shellfish. Park *et al.* (1998) subsequently isolated ten bacteriophages of *L. garvieae* from diseased fish, seawater and sediments by a simple enrichment procedure. These phages varied in their ability to infect *L. garvieae* strains thereby providing a basis for phage typing of *L. garvieae*.

Nakai *et al.* (1999) reported on three of their bacteriophages with respect to the characteristics of environmental and biological stability, fate in the yellowtail and efficacy of one of these phages in the treatment of lactococcosis infections of yellowtail. Environmentally the phages were demonstrated to be stable at temperatures of 5-37°C, in sterile water of 0 to 70 parts per thousand salinity, and at pH 3.5, exhibiting a slow decline in titre of one to two orders of magnitude over eight weeks. Biologically the bacteriophages were stable in moist-pelleted feed for at least three hours, in yellowtail serum for at least three hours, and in extracts of the yellowtail gastrointestinal tract for at least 30 minutes at pHs greater than 3.5.

Investigation of the fate of 3.2×10^7 pfu of bacteriophage injected i.p. revealed their presence in the spleen of the yellowtail from three to 24 h post-injection but they were undetectable in the spleen at two days post-injection. If 3.2×10^7 phage were injected i.p. simultaneously with 5.0×10^8 cfu of *L. garvieae*, phage were still present in the spleen of the yellowtail at five days post-injection at a titre of 1.6×10^2 pfu/g of tissue. When phage were incorporated in a dry-pelleted diet and fed to yellowtail to provide a dose of 2.0×10^7 pfu, the phage were detectable in the stomach and the intestinal tract at 3 and 10 h post-feeding but not at 24 h post-feeding. When the phage and *L. garvieae* were both incorporated in a dry-pelleted ration and fed to provide doses of 2.0×10^7 pfu of phage and 5.0×10^8 cfu of *L. garvieae*, the phage was detectable in the stomach at 3 and 10 h post-feeding but not at 24 h post-feeding. It was detectable at 3, 10 and 24 h post-feeding in the intestine at titres of 10^1 to 10^3 pfu/g of digesta. High concentrations of phage were found in the excreted feces from both types of feed pellets.

The efficacy of the *L. garvieae* bacteriophage, PLg-16, in preventing mortality was assessed by two disease models: an injection-challenge model to assess the efficacy of injected phage in preventing or reducing mortality; and an anal intubation exposure model to assess the efficacy of pre-feeding of phages as a means of preventing the occurrence of disease and mortality.

The injection-challenge model of the disease consisted of i.p. injection of 6.3×10^8 cfu of *L. garviea*. The efficacy of the bacteriophage in preventing mortality was assessed by injecting 3.2×10^7 pfu (M.O.I. of 0.05) i.p. either simultaneously with the *L. garvieae* challenge or with a delay of 1 h or 24 h post-challenge. The control consisted of no phage intervention. The survival of the yellowtail was 100% when phage was injected concurrently with the *L. garvieae* challenge, 80% with a 1 h delay, 50% with a 24 h delay and 10% with no phage intervention. The data demonstrated a statistically-significant reduction in the mortality of the yellowtail from *L. garvieae* infection as a result of therapeutic injection of the bacteriophage. The data also demonstrated that the earlier the intervention the greater the benefit.

The procedure Nakai *et al.* (1999) used to assess the potential of prior exposure of yellowtail to a *L. garvieae* bacteriophage to protect against *L. garvieae* infections consisted of feeding dry-pelleted rations containing either no phage, viable phage, or viable phage and *L. garvieae*. The doses of phage and *L. garvieae* delivered orally in the feed was 2.0×10^7 pfu of phage and 5.0×10^8 cfu of *L. garvieae*, respectively, per fish. The fish were fed the specified rations for 30 minutes and then challenged with 3.2×10^9 cfu of *L. garvieae* by anal intubation. The mortality rate of the yellowtail that did not receive prior exposure to bacteriophage in the feed was 65%. Receiving both phage and *L. garvieae* in the diet reduced the mortality rate to 20% while feeding diet containing the phage alone reduced the mortality to 10%. The data indicate that prior exposure to the bacteriophage protected the fish against mortality caused by infection with *L. garvieae*. It also demonstrated that it is possible to deliver effective concentrations of phage orally.

The research group of Park and Nakai further extended their experience and knowledge on the potential of bacteriophages to control bacterial diseases of farmed fish through research on the bacteriophages of *Pseudomonas plecoglossicida*, the bacterium responsible for hemorrhagic ascites of ayu (*Plecoglossus altivelis*) a relatively new disease that inflicts a high mortality rate (Park *et al.* 2000). In the absence of a chemotherapeutic compound licensed for treating ayu with this disease, Park *et al.* (1997, 1998, 2000) recovered eight phages capable of infecting *P. plecoglossicida* from diseased ayu and from pond culture water. These phages were of two types, one tentatively classified as a member of the family *Myoviridae*, the other a member of *Podoviridae*. All 27 strains of *P. plecoglossicida* tested were infected by both types of phage. Eight other species of bacterial fish pathogens, including three *Pseudomonas* species, were resistant to infection by the phages.

In assessing the ability of these phages as candidates for control of this disease, Park *et al.* (2000) developed an oral-challenge disease model in which the ayu were fed a dry-pelleted ration that had been impregnated with a live culture of *P. plecoglossicida*, providing a challenge dose of 1.5×10^6 cfu. After feeding the *P. plecoglossicida*-impregnated feed for 15 min., duplicate groups of ayu were fed either dry-pelleted ration containing 1.5×10^6 pfu of the two phage types, or the corresponding diet without phage present (control groups). The mean mortality rate of the control group was 65% whereas the presence of the phages reduced the mean mortality to 22.5%. This study was repeated with smaller ayu, withholding the phage treatment for 1 h or 24 h. The second study was done without replication, implying that results cannot be stated with the same level of confidence. However, results demonstrated the same degree of protection with the delay in phage administration as was observed in the original study. *P. plecoglossicida* was isolated from kidney tissue of all dead fish, and also from some survivors in the control group, but not from any of the survivors from the group that received phage. The observation that the tissues of the surviving bacteriophage-treated ayu did not contain *P. plecoglossicida,* whereas the tissues of the surviving control ayu (untreated with the bacteriophage) did contain the pathogen, suggests that the bacteriophage therapy may have been effective in reducing or eliminating a pathogen carrier state of this agent. The bacteria that were isolated from surviving fish remained susceptible to both phages. The authors state that orally-administered phages rapidly appear in the kidney without host bacterial cells as a transport vehicle, although the time that the phage was detectable in the kidney was relatively short.

Based on knowledge gained in research of the bacteriophages of *Lactococcus garvieae* and *Pseudomonas plecoglossicida*, Nakai and Park (2002) cite the following challenges to the application of bacteriophage therapy to aquaculture that have yet to be addressed:

1. the need to find the most aggressive strain of bacteriophage to maximize the therapeutic effect;
2. bacteriophages with a narrow host-strain specificity, or being strain-specific, are poor candidates for application to control of bacterial species with a diversity of strains;

3. bacteriophage-resistant bacteria can appear rapidly *in vitro* but this was not experienced in the *in vivo* studies; the lower rate of development of phage resistance *in vivo* may be simply a reflection of the additional constraints that the *in vivo* environment places on the survival of the bacteriophage mutants;
4. many, but not all, phage-resistant variants are less virulent to the host than the wild type; the fact that many but not all of the phage-resistant mutants are less pathogenic to the animal hosts than the wild-type may reflect the fact that there is a greater number of genetic possibilities for phage-resistant mutants with pathogenicity less than the wild type than there are genetic possibilities for resistant mutants with pathogenicity equal to or greater than the wild type;
5. bacteriophage-neutralizing antibodies may be produced that could be an obstacle to the use of phage therapy in recurrent infections; no such problem was detected in the research programs on bacteriophages with yellowtail or ayu; and,
6. there are risks that phages might mediate genetic exchange among bacteria; the authors state the opinion that this is unlikely provided only bacteriophages with either narrow host specificity, or at least with species specificity, are used in phage therapy.

Bacteriophages and Aeromonas salmonicida

One of the earliest published records of the application of bacteriophages to bacterial fish pathogens was that of Paterson *et al.* (1969) to *Aeromonas salmonicida*. Subsequent reports on the investigation of phages infecting *A. salmonicida* include those of Popoff (1971), Rodgers *et al.* (1981), Ackermann *et al.* (1985), Hidaka and Kawaguchi (1986), Stevenson *et al.* (1994) and Roberts *et al.* (2002). These studies have provided considerable documentation of the existence of, and the characteristics of, bacteriophages of *A. salmonicida* subspecies *salmonicida*. It is unclear from these publications whether the host range of the *A. salmonicida* phages extends to any of the various atypical *A. salmonicida* subspecies.

Rodgers *et al.* (1981) and Olivier (1992) have applied a selection of the *A. salmonicida* phages to the typing of *A. salmonicida* with Olivier (1992) including some atypical *A. salmonicida* isolates. However, the application of the phages to the atypical *A. salmonicida* bacteria required that the cultures be pre-processed in order for any of the isolates to be susceptible to the selection of phages available (Gilles Olivier, DFO Moncton, pers. comm.). This may be consistent with the role of the S-layer (A-layer) protein of virulent *A. salmonicida* isolates in the protection of the bacterial cell against bacteriophages, proteases and complement (Kay and Trust 1991).

In spite of a considerable body of published information on the *in vitro* characteristics of bacteriophages of *A. salmonicida*, and the international importance to salmonid aquaculture of the disease caused by this organism, only one reference has been located on the application of bacteriophages to control furunculosis in rainbow trout (Anonymous 1987). The author of this article reported that a bacteriophage of *A.*

salmonicida was successful in preventing the occurrence of furunculosis in farmed rainbow trout in Japan.

CONCLUSIONS

Published reports on investigations on the potential for bacteriophages to control bacterial diseases, including those of farmed fish, support the following statements:

1. properties established to date of the bacteriophages of bacterial pathogens of finfish are identical to those of phages of bacterial pathogens of terrestrial animals, including humans;
2. comparison of antibiotics and bacteriophages as therapeutants of bacterial disease as summarized in Table 2 applies to the use of these agents against bacterial pathogens of aquacultured finfish;
3. use of bacteriophages to treat bacterial diseases of aquacultured species has the added advantage of reduction of the environmental load of the pathogen as the phages are as effective in the environment as they are in the fish;
4. there is a suggestion that bacteriophage therapy may be effective in reducing or eliminating carrier states of bacterial pathogens of finfish; and,
5. the combined characteristics of bacteriophages as therapeutic agents for bacterial diseases of aquacultured species indicate that there should be a serious effort to assess their potential using various models of bacterial disease of salmonids.

There are regulatory and infrastructure considerations that need to be addressed. This will be the direction of a future literature review and discussions with federal, provincial and international agencies concerned with consumer and environmental safety considerations.

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Appendix A: Some of the major human phage therapy studies performed in Poland and the former Soviet Union. (Modified from Sulakvelidze *et al.* 2001).

Infection(s)	Etiologic agent(s)	Comments	Reference
Bacterial dysentery	*Shigella* sp.	*Shigella* phages were successfully used for prophylaxis of bacterial dysentery	Babalova, E.G., Katsitadze, K.T., Sakvarelidze, L.A., Imnaishvili, N.S., Sharashidze, T.G., Badashvili, V.A., Kiknadze, G.P., Meiparani, A.N., Gendzekhadze, N.D., Machavariani, E.V., Gogoberidze, K.L., Gozalov, E.I. and Dekanosidze, N.G. 1968. Preventive value of dried dysentery bacteriophage. Zh. Mikrobiol. Epidemiol. Immunobiol. 2: 143-145.
Infections of skin and nasal mucosa	*Klebsiella ozaenae, K. rhinoscleromatis,* and *K. pneumoniae*	Adapted phages were reported to be effective in treating *Klebsiella* infections in all of the 109 patients.	Bogovazova, G.G., Voroshilova, N.N., Bondarenko, V.M., Gorbatkova, G.A., Afanas'eva, E.V., Kazakova, T.B., Smirnov, V.D., Mamleeva, A.G., Glukharev, I.A., Erastova, E.I., Krylov, I.A., Ovcherenko, T.M., Baturo, A.P., Yalsky, G.V. and Arefyeva, N.A. 1992. Immunobiological properties and therapeutic effectiveness of preparations from *Klebsiella* bacteriophages. Zh. Mikrobiol. Epidemiol. Immunobiol. 3: 30-33.
Suppurative skin infections	*Pseudomonas* sp., *Staphylococcus* sp., *Klebsiella* sp., *Proteus* sp. and *E. coli*	Thirty-one patients having chronically infected skin ulcers were treated orally and locally with phages. The success rate was 74 percent.	Cislo, M., Dabrowski, B., Weber-Dabrowska, B. and Woyton, A. 1987. Bacteriophage treatment of suppurative skin infections. Arch. Immunol. Ther. Exp. 2: 175-183.
Lung and pleural infections	*Staphylococcus* sp., *Streptococcus* sp. , *Proteus* sp. and *E. coli*	Phages were successfully used together with antibiotics to treat lung and pleural infections in 45 patients	Ioseliana, G.D., Meladze, G.D., Chkhetiia, N.S., Mebuke, M.G. and Kiknadze, N.I. 1980. Use of bacteriophage and antibiotics for prevention of acute post-operative empyema in chronic suppurative lung diseases. Grudn. Khir. 6: 63-67.

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Appendix A: (Cont'd.)

Infection(s)	Etiologic agent(s)	Comments	Reference
Post-operative wound infections in cancer patients	*Staphylococcus* sp. and *Pseudomonas* sp.	A total of 131cancer patients having post-surgical wound infections participated in the study. Of these 65 patients received phages and the rest received antibiotics. Phage treatement was reported successful in 82 percent of the cases, and antibiotic treatment was reported successful in 61 percent of the cases.	Kochetkova, V.A., Mamontov, A.S., Moskovtseva, R.L., Erastova, E.I., Trofimov, E.I., Popov, M.I., and Dzhubalieva, S.K. 1989. Phagotherapy of post-operative suppurative, inflammatory complications in patients with neoplasms. So. Med. 6: 23-26.
Various infections	*Staphylococcus* sp., *Klebsiella* sp., *E. coli*, *Pseudomonas* sp. and *Proteus* sp.	Immunogenicity of therapeutic phages was analyzed in 57 patients. The authors concluded that the phages' immunogenicity did not impede therapy.	Kucharewicz-Krukowska, A. and Slopek, A. 1987. Immunogenic effect of bacteriophage in patients subjected to phage therapy. Arch. Immunol. Ther. Exp. 5: 553-561.
Recurrent subphrenic abscess	*E. coli*	Recurrent subphrenic abscess (after stomach resection) caused by an antibiotic resistant strain of *E. coli* was successfully treated with phages.	Kwarcinski, W.B., Lazarkiewicz B., Weber-Dabrowska, B. Rudnicki, J., Kaminski, K. and Sciebura, M. 1994. Bacteriophage therapy in the treatment of recurrent subphrenic and subhepatic abscess with jejunal fistula after stomach ressection. Pol. Tyg. Lek. 49:535.
Intestinal dysbacteriosis	*E. coli* and *Proteus* sp.	Phages were successfully used together with bifidobacteria to treat antibiotic-associated dysbacteriosis in 500 low birth weight infants.	Litvinova, A.M., Chtetsova, V.M. and Kavtreva, I.G. 1978. Evaluation of the efficacy of the use of *E. coli-Proteus* bacteriophage in intestinal dysbacteriosis in premature infants. Vopr. Okhr. Materin. Det. 9: 42-44.
Lung and pleural infections	*Staphylococcus* sp.	Phages were used to treat 223 patients having lung and pleural infections. Results were compared to117 cases were antibiotics were used. Full recovery was reported in 82 percent of the patients in the phage-treated group, as compared to 64 percent of the patients in the anti-biotic treated group.	Meladze, G.D., Mebuke, M.G., Chkhetia, N.S., Kiknadze, N.I., Koguashvili, G.G., Timosuk, I.I., Larionova, N.G. and Vasadze, G.K. 1982. The efficacy of staphylococcal bacteriophage in treatment of purulent diseases of the lungs and pleura. Grudn. Khir. 1: 53-56.

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Appendix A: (Cont'd.)

Infection(s)	Etiologic agent(s)	Comments	Reference
Bacterial dysentry and salmonellosis	*Shigella* sp. and *Salmonella* sp.	The effectiveness of treating salmonellosis using phages and a combination of phages and antibiotics was examined. The combination of phages and antibiotics was reported to be effective in treating cases where antibiotics alone were ineffective.	Miliutina, L.N. and Vorotyntseva, N.V. 1993. Current strategy and tactics of etiotropic therapy of acute intestinal infections in children. Antibiot. Khimioter. 1: 46-53
Inflammatory urologic disease	*Staphylococcus* sp., *E. coli* and *Proteus* sp.	Adapted phages were used to treat acute and chronic urogenital inflammation in 46 patients. The efficacy of phage treatment reported was 92 percent (marked clinical improvement) and 84 percent (bacteriological clearance).	Perepanova, T.S., Darbeeva, O.S., Kotliarova, G.A., Kondrat'eva, E.M., Maiskaia, L.M., Malysheva, V.F., Baiguzina, F.A. and Grishkova, N.V. 1995. The efficacy of bacteriophage preparations in treating inflammatory urologic diseases. Urol. Nefrol. 5: 14-17
Peritonitis, osteomyelitis, lung abscesses and post-surgical wound infections	*Staphylococcus* sp., *Streptococcus* sp. and *Proteus* sp.	Phages administered subcutaneously or through surgical drains in 236 patients having antibiotic-resistant infections eliminated the infections in 92 percent of the patients.	Sakandelidze, V.M. and Meipariani, A.N. 1974. Use of combined phages in suppurative inflammatory diseases. Zh. Mikrobiol. Epidemiol. Immunobiol. 6: 135-136.
Infectious allergoses (rhinitis, pharyngitis, dermatitis, and conjunctivitis)	*Staphylococcus* sp., *Streptococcus* sp., *E. coli, Proteus* sp., enterococci and *P. aeurginosa*	A total of 1,380 patients having infectious allergoses were treated with phages (360 patients), antibiotics (404 patients), or a combination of phages and antibiotics (576 patients). Clinical improvement was reported in 86, 48 and 83 percent of the cases, respectively.	Sakandelidze, V.M. 1991. The combined use of phages and antibiotics in different infectious allergoses. Vrach. Delo 3: 60-63
Gastrointestinal tract, skin, head and neck infections	*Staphylococcus* sp., *Pseudomonas* sp., *E. coli, Klebsiella* sp. and *Salmonella* sp.	A total of 550 patient were treated with phages. The overall success rate of phage treatment reported was 92 percent.	Slopek, S., Weber-Dabrowska, B., Dabrowski, M. and Kuchaerwicz-Krukowska, A. 1987. Results of bacteriophage treatment of suppurative bacterial infections in the years 1981-1986. Arch. Immunol. Ther. Exp. 35: 569-583.

Appendix A: (Cont'd.)

Infection(s)	Etiologic agent(s)	Comments	Reference
Cerebrospinal meningitis	*Klebsiella pneumoniae*	Orally administered phages were used successfully to treat meningitis in a newborn (after antibiotic therapy failed).	Stroj, L.B., Weber-Dabrowska, B., Partyka, K., Mulczyk, M. and Wojcik, M. 1999. Successful treatment with bacteriophage in purulent cerebrospinal meningitis in a newborn. Neurol. Neurochir. Pol. 3: 693-698.
Bacterial dysentry	*E. coli* and *Proteus* sp.	Phages were used together with bifidobacteria to treat bacterial dysentry in 59 immunosuppressed leukemia patients. The superiority of treatment with phage-bifidobacteria over antibiotics was reported.	Tolkacheva, T.V., Abakumov, E.M., Martynova, V.A. and Golosova, T.V. 1981. Correction of intestinal dysbacteriosis with biological preparations in acute leukemia. Probl. Gematol. Pereliv. Krovi 7: 29-33.
Suppurative infections	*Staphylococcus* sp. and various Gram-negative bacteria	Orally administered phages were used successfully to treat 56 patients. The phages were found to reach the patients' blood and urine.	Weber-Dabrowska, B., Dabrowski, M. and Slopek, S. 1987. Studies on bacteriophage penetration in patients subjected to phage therapy. Arch. Immunol. Ther. Exp. 35: 563-568.
Suppurative surgical infections	*Staphylococcus* sp., *Streptococcus* sp., *E. coli* and *Proteus* sp.	The superiority of adapted phages (phages selected against bacterial strains isolated from individual patients) over commercial phage preparations was reported in treating 60 patients having suppurative infections.	Zhukov-Vereezhnikov, N.N., Peremitina, L.D., Berillo, E.A., Komissarov, V.P., Bardymov, V.M, Khvoles, A.G. and Ugryumov, L.B. 1978. A study of the therapeutic effect of bacteriophage agents in a complex treatment of suppurative surgical diseases. Sov. Med. 12: 64-66.